Supporting Material

Additional Material and Methods

Our phylogenetic analyses are based upon 97 volvocine terminal units (strains and species), most of these species being well characterized phenotypically and genetically. However, two taxa, *G. pectorale* Russia and *Volvox perglobator* Tucson, had not yet been well characterized, so we describe how genotypic and phenotypic data was collected from these taxa.

Culture Conditions

Two strains (*Gonium pectorale* Russia and *Volvox perglobator* Tucson) were grown in standard *Volvox* medium (SVM) at 25°C on a 16:8 hour light:dark cycle at approximately 35 μmol photons/m^2/s.

DNA Preparation, PCR Cloning, and Sequencing

Five chloroplast genes (ATP synthase beta-subunit, *atpB*; P700 chlorophyll a-apoprotein A1, *psaA*; P700 chlorophyll a-apoprotein A2, *psaB*; photosystem II CP43 apoprotein, *psbC*; and the large subunit of rubisco, *rbcL*) were sequenced in two taxa (*G. pectorale* Russia and *V. perglobator* Tucson). Genomic DNA was prepared according to Miller and Kirk (1999). PCR reactions were performed using 2X Phusion HF Master Mix (Thermo Scientific, Waltham, MA). Cycling conditions were 98°C for 2 minutes, followed by 37 cycles of 98°C/10 seconds, variable annealing temperature (47.4–60.1°C) for 20 seconds, 72°C for variable extension time (15-150 seconds), then a finishing step of 72°C for 5 minutes (Table S1, available in Dryad: doi:). PCR amplifications intended for sequencing were performed in at least three separate reactions and then combined before sequencing to reduce the possibility of PCR errors. DNA sequencing was performed by the University of Arizona Genetics Core using Applied Biosystems 3730 DNA Analyzers (Waltham, MA).

Identification of Character States

For *Gonium pectorale* Russia, the maximum reproductive cell number was measured using a Benchtop B3 Series FlowCam model VS-IV (Fluid Imaging Technologies, Scarborough ME). A 20X objective was used, on AutoImage Mode, to image over 500 colonies of exponential phase *G. pectorale* Russia. *Gonium pectorale* Russia has a maximum of 16 cells, all of which are reproductive. The maximum colony length, 85 μm, was evaluated based on 25 images using a Nikon Eclipse Ti-E (Nikon, Tokyo, Japan). These values are consistent with other strains of *G. pectorale* (Table S4). This strain does not show sexual activity in nitrogen-deficient medium (SVM with urea omitted and 0.5 M $CaCl_2$ replacing $Ca(NO_3)_2$), possibly caused by a decline in mating efficiency due to long-term culture (Hamaji et al. 2013).

Cultures of *Volvox perglobator* Tucson were isolated from a water fountain in Reid Park, Tucson, Arizona in October 2012 and September 2014 (GPS coordinates: 32° 12' 35.6" N, 110° 55' 21.2" W). This population was identified as *Volvox perglobator* based on heterothallic, dioecious mating and straight, blunt-tipped zygotes (Isaka et al. 2012). Single colonies were isolated and grown until sexuality was naturally induced. Every isolate produced either sperm or eggs, never both (heterothallic and dioecious).

Male and female strains were observed using a Nikon SMZ800 stereomicroscope (Nikon, Tokyo, Japan) throughout their lifecycle. When male and female strains were mixed, sex was oogamous with internal fertilization, producing zygotes. The number of somatic cells present in a sample of 20 asexual colonies was evaluated by counting the number of cells on the circumference (n) and inferring the total number of somatic cells (N) by assuming cells are hexagonally arranged (Smith 1944). Setting the surface area of a sphere equal to the surface area of N hexagonal cells and using the equivalent formula for circumference to solve for N (Janet 1912), $N = \frac{2}{\pi\sqrt{3}}n^2$. We observed an average of 3,800 somatic cells and a maximum of 7,500 somatic cells (cell numbers are rounded following convention and reflecting the approximate nature of this approach). The maximum reproductive cell number was measured using a Benchtop FlowCam model VS-IV (Fluid Imaging Technologies, Scarborough ME). A 4X objective was used, on Trigger Mode, to image 250 colonies of exponential phase *V. perglobator* Tucson. Asexual colonies have 1–11 gonidia (reproductive cells/structures), with 4 gonidia on average. A maximum colony length of 657 μm, measured using a Benchtop FlowCam (Fluid Imaging Technologies, Scarborough, ME), was determined from a sample of 750 mature adult colonies. The female strain spontaneously differentiated in older cultures with 18–79 eggs, with 53 eggs on average.

Figure S1. Life cycles of two volvocine green algae, the unicellular and isogamous *Chlamydomonas reinhardtii* and the multicellular and oogamous *Volvox carteri*. All sexual traits studied here are absent in *Chlamydomonas reinhardtii* and are present in *Volvox carteri*. In both species, asexual reproduction includes growth, multiple rounds of cell division, and hatching into daughter offspring. Following sexual differentiation, fusion and fertilization of gametes produces a dormant, diploid zygospore. Only this zygospore can withstand detrimental environmental conditions including heat, freezing, and desiccation. When environmental conditions improve, the zygospore hatches and enters the asexual lifecycle.

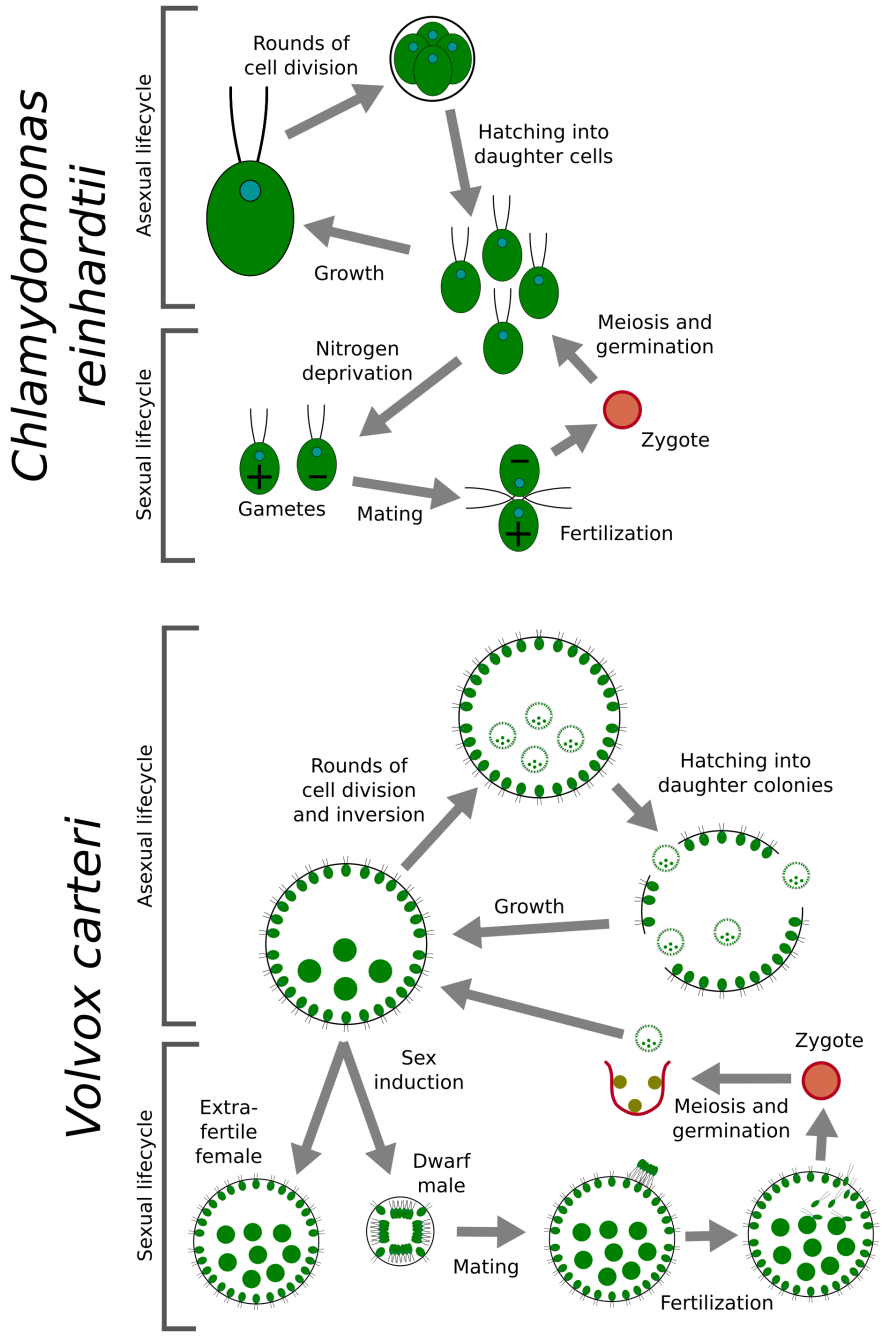

Figure S2. Plots of ln-transformed anisogamy ratio (defined by macrogamete volume divided by microgamete volume, a measure of gamete dimorphism) and (A) rounds of cell division, (B) percent of somatic cells, and (C) ln-transformed body length. Relative size of the data points (tips of the phylogeny) are scaled to indicate the number of species at that coordinate (from 1 to 37). Phylogenetic regression lines and statistics (red) and simple linear regression and statistics (blue) are shown.

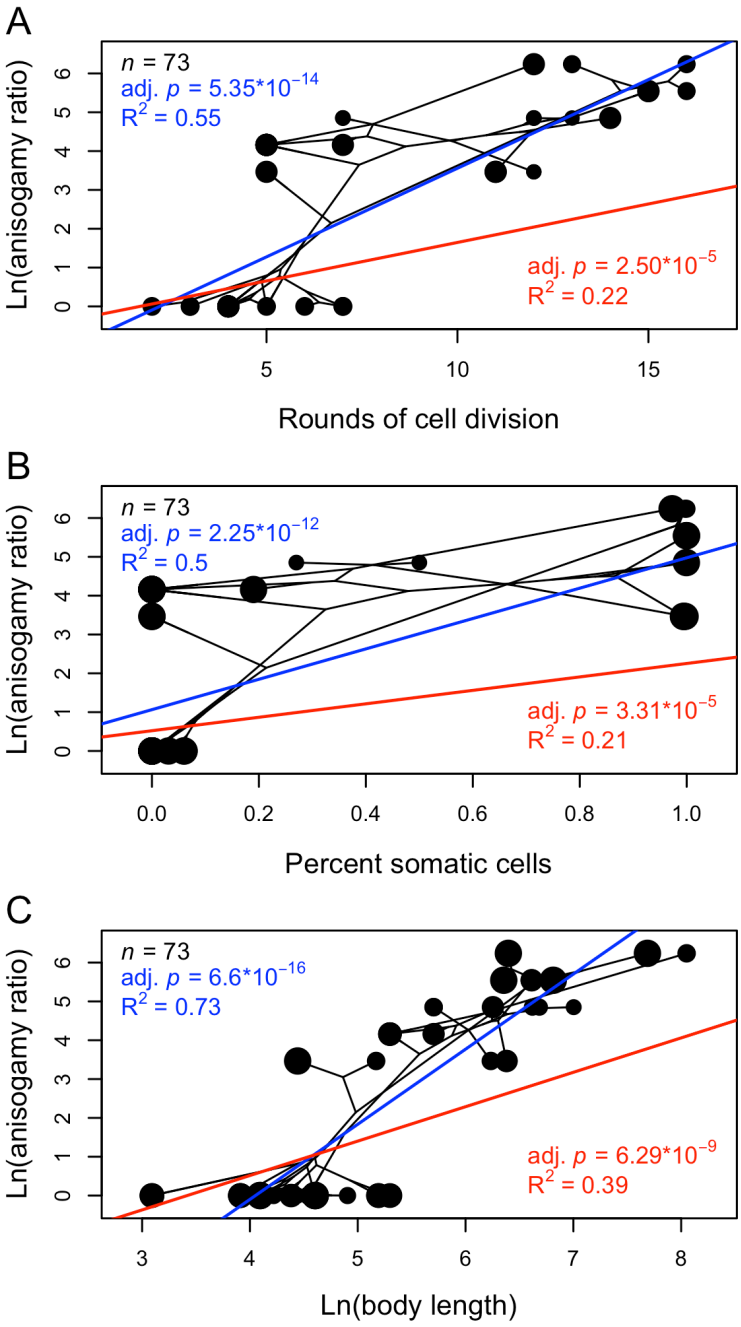

Table S1. PCR primers used in this study. *indicates a reverse primer.

Primer name	Target locus	Sequence (5' -> 3')	Source
atpB-1436R*	*atpB*	TGTTACCTACTAARTAGAAAGATTGTT	This study
atpB-171F	*atpB*	AGAAATGGCTGTWACTTGTGA	This study
atpB-F7	*atpB*	GATAAYATTTTCCGWTTYGT	Nozaki et al. (1995)
atpB-R5*	*atpB*	ACNCCAGATTCTTTCATTTC	Nozaki et al. (1995)
psaA-1093F	*psaA*	GTATTATTTGCTCGTAGCTC	This study
psaA-2096R*	*psaA*	CTTTCAATAAGTTCTTGCCAGTA	This study
psaA-323R*	*psaA*	TGGTGAGCAGTATCACTTAACC	This study
psaA-532F	*psaA*	TGGTTCCACTATCACAAAGC	This study
psaB-1054F	*psaB*	GGTTTCCAYACTTTAGGTCTTTATG	This study
psaB-1602R*	*psaB*	CCAATAGTGTTAAGCATCCAG	Herron and Michod (2008)
psaB-31F	*psaB*	AGCWTGGCAAGGTAACTTT	Herron and Michod (2008)
psaB-907R*	*psaB*	NGCACCACACATDATRAA	Herron and Michod (2008)
psbC-1164R*	*psbC*	CGGAGCATGTGTCATRTATTC	This study
psbC-197F	*psbC*	AAAAACCAATGTATGARCAAGG	This study
psbC-F1	*psbC*	TCAACCAATGGGTTATGAAACA	This study
psbC-F2	*psbC*	GCGGTGGGCCAAATTAAAG	This study
psbC-F3	*psbC*	CTTACGGAAATGTGGTTTTTC	This study
psbC-R1*	*psbC*	CTTCATATCCTCACGCCCTTAG	This study
psbC-R2*	*psbC*	CAGGCGCCCAAGTATCATAAA	This study
psbC-R3*	*psbC*	TGAAGTTCAGACTATGTCTTC	This study
rbcL-1F	*rbcL*	ATGGTTCCACAAACAGAAAC	Nozaki et al. (1995)
rbcL-890R*	*rbcL*	ACGGTGGATGTGAAGAAGTAAA	This study
rbcL-F214	*rbcL*	GACGGTTTAACTAGCCTTGAC	This study
rbcL-F320	*rbcL*	TATTCGAAGAAGGTTCAGTAAC	Nozaki et al. (1995)
rbcL-F499	*rbcL*	CGTGGTCTTTTAGGTTGTACAAT	This study
rbcL-F650	*rbcL*	GTTTCCTTTTCGTAGCTGAAGC	Nozaki et al. (1995)
rbcL-R1181*	*rbcL*	AAGATTTCAACTAAAGCTGGCA	Nozaki et al. (1995)
rbcL-R395*	*rbcL*	GCACGTAAAGCTTTGAAACC	Nozaki et al. (1995)
rbcL-R582*	*rbcL*	ACGTAAACACTCATAAACAGC	This study
rbcL-R783*	*rbcL*	ACCTAATTCTTTAGCACATTGAC	This study
rbcL-R803*	*rbcL*	TCGTGCATAATAATAGGTACAC	Nozaki et al. (1995)

Table S2. Genbank accession numbers of chloroplast genes for ingroup and outgroup taxa.

Available on Dryad: doi:

Table S3. The best partition and nucleotide substitution scheme as determined by PartitionFinder version 2.1.1 (Lanfear et al. 2016). "Position" refers to codon position.

Partition	Substitution model
atpB position 1 *rbcL* position 1	GTR+Γ+I
atpB position 2 *psaB* position 2	GTR+Γ+I
atpB position 3	GTR+Γ+I
psaA position 1	GTR+Γ+I
psaA position 2 *psbC* position 2	GTR+Γ+I
psaA position 3	GTR+Γ+I
psaB position 1	GTR+Γ+I
psaB position 3 *psbC* position 3	GTR+Γ+I
psbC position 1	GTR+Γ+I
rbcL position 2	GTR+Γ+I
rbcL position 3	GTR+Γ+I

Table S4. Continuous multicellularity metrics and discrete character states for sexual traits and associated references for volvocine taxa. For heterothallic species, only one strain of a mating pair has been given.

Available on Dryad: doi:

Table S5. Model fit and parameter estimates for analyses of discrete traits. For phylogenetic signal, the D value was calculated (D < 0 indicates stronger phylogenetic signal than a Brownian motion model; D = 0 indicates Brownian motion; D = 1 indicates random, non-phylogenetic distribution of states among taxa). Significant *P*-values associated with a given model indicate that the model is rejected. For discrete traits, the first state listed in the column header is state 0, and subsequent states are state 1 or state 2. The value q is the transition rate between the two states (ancestral state on the left, derived state on the right). Bayes factors (BF), taking phylogenetic uncertainty into account, were estimated based on twice the difference between the highest harmonic mean log likelihood for each model (ER, equal rates; SYM, symmetric rates; ARD, all rates different), calculated from nine independent MCMC runs for each model. Interpretation of Bayes factors is as follows: 0 to 2 barely worth mentioning, 2 to 6 positive, 6 to 10 strong, >10 very strong (Kass and Raftery 1995).

		All/reduced meiotic products	Isogamy/ anisogamy	Isogamy/ oogamy	Isogamy/ anisogamy/ oogamy	External/internal fertilization	Absence/ presence of extra-fertile females	Absence/ presence of dwarf males
Log likelihood values	D	-1.569	-1.017	-0.757	-	-1.14	-0.695	-1.276
	P(D=0)	0.995	1	0.995	-	1	0.995	0.965
	P(D=1)	0	0	0	-	0	0	0
	ER	-10.41	-	-	-27.16	-9.02	-19.63	-14.29
	SYM	-	-	-	-23.64	-	-	-
	ARD	-8.17	-	-	-22.81	-8.46	-18.9	-11.62
	Best Model	ARD	-	-	SYM	ER	ER	ARD
	ΔAICc	2.35	-	-	2.78	1	0.63	3.24
	q01	0.45	-	-	0.11	0.21	0.48	0.2
	q10	1.80E-11	-	-	0.11	0.21	0.48	7.79
	q12 = q21	-	-	-	0.64	-	-	-
	q02 = q20	-	-	-	1.39E-08	-	-	-
Highest harmonic mean log likelihood	ER	-11.29	-	-	-28.4	-10.53	-20.69	-14.87
	SYM	-	-	-	-28.37	-	-	-
	ARD	-12.08	-	-	-65.1	-11.32	-21.41	-14.77
	Best model	ARD	-	-	SYM	ER	ER	ARD
	Bayes factor	1.59	-	-	0.06	1.58	1.45	0.19

Table S6. Model fit and parameter estimation for analyses of continuous traits. Phylogenetic signal was assessed using Blomberg's K (2003) and Pagel's λ (Pagel 1999). Statistical significance for these indices was based on comparison to a non-phylogenetic model (K = 0, λ = 0), with a significant *P*-value indicating rejection of the non-phylogenetic model.

		Rounds of cell division	Percent of cells somatic	Ln body length (μm)	Ln zygote diameter (μm)	Ln anisogamy ratio
Blomberg's K	K	1.655	2.483	2.233	1.626	4.117
	P(K=0)	0.001	0.001	0.001	0.001	0.001
Pagel's λ	λ	0.986	1.003	0.983	0.962	1.006
	P(λ=0)	8.53E-38	4.93E-50	1.55E-36	6.19E-23	4.13E-27
Maximum likelihood AICc values	Brownian	380.86	-94.49	-192.35	8.97	-
	Ornstein-Uhlenbeck	380.91	-92.36	-190.22	9.63	-
	λ	378.88	-92.36	-196.91	-2.09	-
	White noise	543.35	113.49	-10.24	97	-
	ΔAICc	1.98	0	4.56	11.06	-
	Best Model	λ	OU and λ	λ	λ	-

Table S7. Correlations between internal fertilization and anisogamy and oogamy using Pagel's test (1994)

Trait Correlation	Likelihood-ratio	*p*-value	adj. *p*-value
Internal fertilization and anisogamy	11.01	0.026	0.026
Internal fertilization and oogamy	12.19	0.016	0.026

Supplemental Literature Cited

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